EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance

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EUCAST subcommittee for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance:
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1. Introduction

These guidelines have been produced partly in response to frequently asked questions from users of EUCAST guidelines and partly on request from the European Centre for Disease Prevention and Control (ECDC), as expert guidance was needed for updating the EARS-Net microbiological manual.

The remit of the EUCAST subcommittee was to develop practical guidelines for detection of specific antimicrobial resistance mechanisms of clinical and/or epidemiological importance. The document has been developed mainly for routine use in clinical laboratories and does not cover technical procedures for identification of resistance mechanisms at a molecular level by reference or expert laboratories. However, much of the content is also applicable for national reference laboratories. Furthermore, it is important to note that the document does not cover screening for asymptomatic carriage (colonization) of multidrug-resistant microorganisms or direct detection in clinical samples.

All chapters in this document contain a definition of the mechanism or specific resistance, an explanation of the clinical and/or public health need for detection of the mechanism or specific resistance, an outline description of recommended methods of detection, and references to detailed descriptions of the methods. The need for identification of resistance mechanism and the level of identification needed for public health or infection control purposes may vary both geographically and temporally depending on the prevalence and heterogeneity of different resistance mechanisms. The guidelines have been developed by conducting PubMed literature searches, and recommendations are based on multi-centre studies or multiple single centre studies. Several methods currently under development have not been included in the guidelines as multi-centre evaluations or multiple single centre evaluations are yet to be completed. Draft versions of these guidelines were subject to wide consultation through EUCAST consultation contact lists, the EUCAST website and ECDC focal point contacts.

We have as far as possible used generic terms for the products presented in the document, but excluding all specific product names would have made some of the recommendations unclear. It should be noted that some resistance mechanisms do not always confer clinical resistance. Hence, while detection of these mechanisms may be relevant for infection control and public health, it may not be necessary for clinical purposes. Consequently for some mechanisms, particularly extended-spectrum β-lactamases and carbapenemases in Gram-negative bacilli, detection of the mechanism does not in itself lead to classification as resistant. Finally, the relevance of searching for several transferable β-lactamases when other enzymes with a broader spectrum have already been detected is dubious.

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2. Carbapenemase-producing Enterobacteriaceae

Importance of detection of resistance mechanism	
Required for antimicrobial susceptibility categorization	No
Infection control	Yes
Public health	Yes

2.1 Definition

Carbapenemases are β-lactamases that hydrolyze penicillins, in most cases cephalosporins, and to various degrees carbapenems and monobactams (the latter are not hydrolyzed by metallo-β-lactamases).

2.2 Clinical and/or epidemiological importance

The problem of dissemination of carbapenemases in Europe dates from the second half of the 1990s in several Mediterranean countries, and was observed mainly in *P. aeruginosa* (1). In the early 2000s, Greece experienced an epidemic of the Verona integron-encoded metallo-β-lactamase (VIM) among *K. pneumoniae* (2) followed by an epidemic related to the *K. pneumoniae* carbapenemase (KPC), which is presently the most common carbapenemase in Europe among Enterobacteriaceae (1). In Greece and Italy around 60 and 15%, respectively, of invasive *K. pneumoniae* are now non-susceptible to carbapenems (3). In other European countries several outbreaks have been reported, but the problem has not been widely observed in invasive isolates (1). Other particularly problematic carbapenemases are the New Delhi metallo-β-lactamases (NDMs), which are highly prevalent on the Indian subcontinent and in the Middle East and have on several occasions been imported to Europe. The OXA-48-like enzymes have caused outbreaks in several European countries and are now spreading rapidly (1).

Carbapenemases are a source of concern because they may confer resistance to virtually all β-lactams, strains producing carbapenemases frequently possess resistance mechanisms to a wide-range of antimicrobial agents, and infections with carbapenemase-producing Enterobacteriaceae are associated with high mortality rates (4-6).

2.3 Mechanisms of resistance

The vast majority of carbapenemases are acquired enzymes, encoded by genes on transposable elements located on plasmids. Carbapenemases are expressed at various levels and differ significantly in both biochemical characteristics and activity against specific β-lactams. The level of expression and properties of the β-lactamase and the frequent association with other resistance mechanisms (other β-lactamases, efflux and/or altered permeability) result in the wide range of resistance phenotypes observed among carbapenemase-producing isolates (7, 8). Decreased susceptibility to carbapenems in Enterobacteriaceae may, however, also be caused by either

extended spectrum β-lactamases (ESBL) or AmpC enzymes combined with decreased permeability due to alteration or down-regulation of porins (9).

Most carbapenemase-producers are resistant to extended-spectrum (oxyimino) cephalosporins (10). Isolates producing such enzymes may have decreased susceptibility to carbapenems, but with some of these enzymes (OXA-48-like enzymes) the organisms may appear fully susceptible to cephalosporins. However, many of these isolates now also express cephalosporin-hydrolyzing enzymes, such as CTX-Ms, and are then also resistant to cephalosporins. Carbapenemases are considered to be of high epidemiological importance, particularly when they confer decreased susceptibility to any of the carbapenems (imipenem, meropenem, ertapenem and doripenem), i.e. when the MICs are above the epidemiological cut-off (ECOFF) values defined by EUCAST (11).

2.4 Recommended methods for detection of carbapenemases in Enterobacteriaceae

2.4.1 Screening for carbapenemase-production

Carbapenem MICs for carbapenemase-producing Enterobacteriaceae may be below the clinical breakpoints (10, 11, 13). However, the ECOFF values as defined by EUCAST can be used to detect carbapenemase-producers. Meropenem offers the best compromise between sensitivity and specificity in terms of detecting carbapenemase-producers (10, 14). Ertapenem has excellent sensitivity but poor specificity, especially in species such as *Enterobacter* spp., due to its relative instability to extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases in combination with porin loss (10). Appropriate cut-off values for detecting putative carbapenemase-producers are shown in Table 1. It should be noted that in order to increase specificity, imipenem and ertapenem screening cut-off values are one-dilution step higher than the currently defined ECOFFs.

Table 1. Clinical breakpoints and screening cut-off values for carbapenemase-producing Enterobacteriaceae (according to EUCAST methodology).

Carbapenem	MIC (mg/L)		Disk diffusion zone diameter (mm) with 10 µg disks	
	S/I breakpoint	Screening cut-off	S/I breakpoint	Screening cut-off
Meropenem[1]	≤2	>0.12	≥22	<25[2]
Imipenem[3]	≤2	>1	≥22	<23
Ertapenem[4]	≤0.5	>0.12	≥25	<25

[1]Best balance of sensitivity and specificity

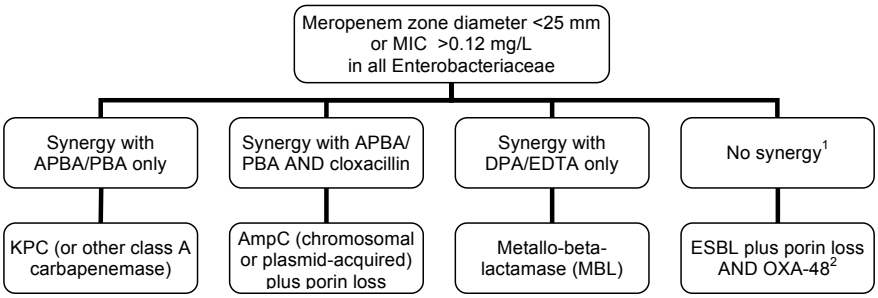
[2]In some cases zone diameters for OXA-48-producers are up to 26 mm, so <27 mm may be used as a screening cut-off in countries where OXA-48 is endemic, but at the expense of lower specificity.

[3]With imipenem, the separation between the wild-type and carbapenemase-producers is relatively poor. Imipenem is therefore not recommended for use as a stand-alone screening test compound.
[4]High sensitivity but low specificity, and therefore not recommended for routine use.

2.4.2 Methods for confirmation of carbapenemase-production

Following detection of reduced susceptibility to carbapenems in routine susceptibility tests, phenotypic methods for detection of carbapenemases should be applied. The combination disk test has the advantage of being well-validated in studies and is also commercially available (MAST, UK; Rosco, Denmark) (15-17). The disks or tablets contain meropenem +/- various inhibitors that are detailed in section 2.4.3. In brief, boronic acid inhibits class A carbapenemases and dipicolinic acid inhibits class B carbapenemases. There is no currently available inhibitor for class D carbapenemases. Cloxacillin, which inhibits AmpC β-lactamases, has been added to the tests to differentiate between AmpC hyperproduction plus porin loss and carbapenemase-production. The algorithm for interpretation of these inhibitor tests is outlined in Figure 1. The main disadvantage with these methods is that they take 18 hours (in practice overnight incubation), for which reason novel rapid methods are now being explored.

Figure 1. Algorithm for carbapenemase detection.



Abbreviations: APBA=aminophenyl boronic acid, PBA=phenyl boronic acid, DPA=dipicolinic acid, EDTA=ethylenediaminetetraacetic acid (all of them β-lactamase inhibitors added to disks or tablets containing meropenem in combination disk testing assays)
[1] Combination of KPC and MBL may not show synergy but isolates are normally highly resistant to carbapenems. They are easiest to detect with molecular methods.
[2] High-level temocillin resistance [MIC >32 mg/L (12, 18), tentative zone diameter <11 mm with temocillin 30 µg disk (17)] are phenotypic indicators of OXA-48 production, which should be considered in the absence of synergy with inhibitors of class A and B carbapenemases.

There are now several more rapid alternatives to the combination disk method. Analysis of carbapenem hydrolysis with MALDI-TOF MS (19) has been reported to confirm carbapenemase production in a few hours, and the Carba NP test (20, 21) can confirm carbapenemase production even more rapidly. However, of these tests there is published evidence only for the Carba NP test beyond the centre where it

was developed. One publication indicates high sensitivity and specificity (22), whereas one publication observed problems of sensitivity for isolates with mucoid phenotype and for some Enterobacteriaceae producing OXA-48 (23).

Several genotypic approaches have been reported based on PCR techniques (24). These methods, however, have the disadvantage of not being able to identify new β-lactamase variants, and might be considered expensive in some settings (10). Commercial DNA microarray methods are marketed and may increase the user-friendliness of these tests (25), although they cannot overcome general limitations of genotypic techniques. It is recommended that at least reference laboratories have access to genotypic confirmation techniques, although this is not strictly required for surveillance purposes.

2.4.3 Interpretation of phenotypic detection methods

The algorithm in Table 2 differentiates between metallo-β-lactamases, class A carbapenemases, class D carbapenemases and non-carbapenemases (ESBL and/or AmpC plus porin loss). The tests can be done with the EUCAST disk diffusion method for non-fastidious organisms. Commercial tests should be set up according to the manufacturer's instructions for each test.

At present there are no available inhibitors for OXA-48-like enzymes. Temocillin high-level resistance (MIC >32 mg/L) has been proposed as a phenotypic marker for OXA-48-like carbapenemase producers (12, 17, 18). However, this marker is not specific for OXA-48-type carbapenemases as other resistance mechanisms might confer this phenotype. The presence of OXA-48-like enzymes therefore has to be confirmed with a genotypic method.

Use of the modified cloverleaf (Hodge) test is not recommended as results are difficult to interpret, the specificity is poor, and in some cases the sensitivity is also suboptimal (10). Some novel modifications of the technique have been described, but they are cumbersome for use in routine clinical laboratories and do not solve all problems of sensitivity and specificity.

Table 2. Interpretation of phenotypic tests (carbapenemases in **bold type**) by diffusion methods with disks or tablets. The exact definitions of synergy are provided in package inserts for the various commercial products.

β-lactamase	Synergy observed as increase in zone diameter (mm) with 10 µg meropenem disk/tablet				Temocillin MIC >32 mg/L or zone diameter <11 mm
	DPA/EDTA	APBA/PBA	DPA+APBA	CLX	
MBL	+	-	-	-	Variable[1]
KPC	-	+	-	-	Variable[1]
MBL + KPC[2]	Variable	Variable	+	-	Variable[1]

OXA-48-like	-	-	-	-	Yes
AmpC + porin loss	-	+	-	+	Variable[1]
ESBL + porin loss	-	-	-	-	No

Abbreviations: MBL=metallo-β-lactamase, KPC=*Klebsiella pneumoniae* carbapenemase, DPA=dipicolinic acid, EDTA=ethylenediaminetetraacetic acid, APBA= aminophenyl boronic acid, PBA= phenyl boronic acid, CLX=cloxacillin.

[1] Temocillin susceptibility test is recommended only in cases where no synergy is detected, in order to differentiate between ESBL + porin loss and OXA-48-like enzymes (12, 17, 18). When other enzymes are present the susceptibility is variable and does not provide any further indication of the β-lactamase present.

[2] There is one report supporting the use of commercial tablets containing double inhibitors (DPA or EDTA plus APBA or PBA) (26), but multi-centre studies or multiple single centre studies are lacking. This combination confers high-level resistance to carbapenems and is rare outside Greece.

2.4.4 The Carba NP test

The principle of this test is that carbapenem hydrolysis will give rise to a pH-change which will result in a colour change from red to yellow with phenol red solution (20,21). The Carba NP test has been validated with bacterial colonies grown on Mueller-Hinton agar plates, blood agar plates, trypticase soy agar plates, and most selective media used in screening for carbapenemase producers. The Carba NP test cannot be performed with bacterial colonies grown on Drigalski or McConkey agar plates. The different steps in the method must be closely followed in order to obtain reproducible results.

2.4.5 Control strains

Appropriate control strains for carbapenemase testing are shown in table 3.

Table 3. Appropriate control strains for carbapenemase testing.

Strain	**Mechanism**
Enterobacter cloacae CCUG 59627	AmpC combined with decreased porin expression
K. pneumoniae CCUG 58547 or *K. pneumoniae* NCTC 13440	Metallo-β-lactamase (VIM)
K. pneumoniae NCTC 13443	Metallo-β-lactamase (NDM-1)
E. coli NCTC 13476	Metallo-β-lactamase (IMP)
K. pneumoniae CCUG 56233 or *K. pneumoniae* NCTC 13438	*Klebsiella pneumoniae* carbapenemase (KPC)
K. pneumoniae NCTC 13442	OXA-48 carbapenemase
K. pneumoniae ATCC 25955	Negative control

2.5 References

1. Cantón R, Akóva M, Carmeli Y, Giske CG, Glupczynski Y, et al. Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe. Clin Microbiol Infect. 2012;18:413-31
2. Vatopoulos A. High rates of metallo-β-lactamase-producing *Klebsiella pneumoniae* in Greece – a review of the current evidence. Euro Surveill. 2008;13(4). doi:pii: 8023
3. European Centres for Disease Prevention and Control (ECDC). Antimicrobial resistance surveillance in Europe 2011. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net)
4. Souli M, Galani I, Antoniadou A, Papadomichelakis E, Poulakou G et al. An outbreak of infection due to β-lactamase *Klebsiella pneumoniae* carbapenemase 2-producing *K. pneumoniae* in a Greek University Hospital: molecular characterization, epidemiology, and outcomes. Clin Infect Dis 2010;50:364–73.
5. Bratu S, Landman D, Haag R, Recco R, Eramo A, Alam M, Quale J. Rapid spread of carbapenem-resistant *Klebsiella pneumoniae* in New York City: a new threat to our antibiotic armamentarium. Arch Intern Med. 2005; 165:1430-5.
6. Marchaim D, Navon-Venezia S, Schwaber MJ, Carmeli Y. Isolation of imipenem-resistant Enterobacter species: emergence of KPC-2 carbapenemase, molecular characterization, epidemiology, and outcomes. Antimicrob Agents Chemother. 2008;52:1413-8
7. Queenan AM, Bush K. Carbapenemases: the versatile β-lactamases. Clin Microbiol Rev 2007;20:440–58
8. Falcone M, Mezzatesta ML, Perilli M, Forcella C, Giordano A et al. Infections with VIM-1 metallo β-lactamase-producing *Enterobacter cloacae* and their correlation with clinical outcome. J Clin Microbiol 2009;47: 3514–9.
9. Doumith M, Ellington MJ, Livermore DM, Woodford N. Molecular mechanisms disrupting porin expression in ertapenem-resistant *Klebsiella* and *Enterobacter* spp. clinical isolates from the UK. J Antimicrob Chemother. 2009;63:659-67
10. Nordmann P, Gniadkowski M, Giske CG, Poirel L, Woodford N, Miriagou V. Identification and screening of carbapenemase-producing Enterobacteriaceae. Clin Microbiol Infect. 2012;18:432-8.
11. European Committee on Antimicrobial Susceptibilty Testing (EUCAST). Website with MIC-distributions. (http://mic.eucast.org/, accessed on 23 December 2012)
12. Glupczynski Y, Huang TD, Bouchahrouf W, Rezende de Castro R, Bauraing C et al. Rapid emergence and spread of OXA-48-producing carbapenem-resistant Enterobacteriaceae isolates in Belgian hospitals. Int J Antimicrob Agents. 2012;39:168-72
13. Tato M, Coque TM, Ruíz-Garbajosa P, Pintado V, Cobo J et al. Complex clonal and plasmid epidemiology in the first outbreak of Enterobacteriaceae infection involving VIM-1 metallo-β-lactamase in Spain: toward endemicity? Clin Infect Dis. 2007;45(9):1171-8.
14. Vading M, Samuelsen Ø, Haldorsen B, Sundsfjord AS, Giske CG. Comparison of disk diffusion, Etest and VITEK2 for detection of carbapenemase-producing *Klebsiella pneumoniae* with the EUCAST and CLSI breakpoint systems. Clin Microbiol Infect. 2011;17:668-74.
15. Giske CG, Gezelius L, Samuelsen Ø, Warner M, Sundsfjord A, Woodford N. A sensitive and specific phenotypic assay for detection of metallo-β-lactamases and KPC in *Klebsiella pneumoniae* with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin. Clin Microbiol Infect. 2011;17:552-6.
16. Doyle D, Peirano G, Lascols C, Lloyd T, Church DL, Pitout JD. Laboratory detection of Enterobacteriaceae that produce carbapenemases. J Clin Microbiol. 2012;50:3877-80.
17. van Dijk K, Voets G, Scharringa J, Voskuil S, Fluit A, Rottier W, Leverstein-Van Hall M, Cohen Stuart J. A disc diffusion assay for detection of class A, B and OXA-48 carbapenemases in Enterobacteriaceae using phenyl boronic acid, dipicolinic acid, and temocillin. Clin Microbiol Infect 2013. In press
18. Hartl R, Widhalm S, Kerschner H, Apfalter P. Temocillin and meropenem to discriminate resistance mechanisms leading to decreased carbapenem susceptibility with focus on OXA-48 in Enterobacteriaceae. Clin Microbiol Infect. 2013;19:E230-2.
19. Hrabák J, Studentová V, Walková R, Zemlicková H, Jakubu V et al. Detection of NDM-1, VIM-1, KPC, OXA-48, and OXA-162 carbapenemases by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol. 2012;50:2441-3

20. Nordmann P, Poirel L, Dortet L. Rapid detection of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis. 2012;18:1503-7.
21. Dortet L, Poirel L, Nordmann P. Rapid Identification of carbapenemase types in Enterobacteriaceae and *Pseudomonas* spp. by using a biochemical test. Antimicrob Agents Chemother. 2012;56:6437-40.
22. Vasoo S, Cunningham SA, Kohner PC, Simner PJ, Mandrekar JN, Lolans K, Hayden MK, Patel R. Comparison of a novel, rapid chromogenic biochemical assay, the Carba NP test, with the modified Hodge test for detection of carbapenemase-producing Gram-negative bacilli. J Clin Microbiol. 2013;51:3097-101.
23. Tijet N, Boyd D, Patel SN, Mulvey MR, Melano RG. Evaluation of the Carba NP test for rapid detection of carbapenemase-producing Enterobacteriaceae and *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2013;57:4578-80.
24. Mililllo M, Kwak YI, Snesrud E, Waterman PE, Lesho E, McGann P. Rapid and simultaneous detection of bla_{KPC} and bla_{NDM} by use of multiplex real-time PCR. J Clin Microbiol. 2013;51:1247-9.
25. Cuzon G, Naas T, Bogaerts P, Glupczynski Y, Nordmann P. Evaluation of a DNA microarray for the rapid detection of extended-spectrum β-lactamases (TEM, SHV and CTX-M), plasmid-mediated cephalosporinases (CMY-2-like, DHA, FOX, ACC-1, ACT/MIR and CMY-1-like/MOX) and carbapenemases (KPC, OXA-48, VIM, IMP and NDM). J Antimicrob Chemother. 2012;67:1865-9.
26. Miriagou V, Tzelepi E, Kotsakis SD, Daikos GL, Bou Casals J, Tzouvelekis LS. Combined disc methods for the detection of KPC- and/or VIM-positive *Klebsiella pneumoniae*: improving reliability for the double carbapenemase producers. Clin Microbiol Infect. 2013;19:E412-5

3. Extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae

Importance of detection of resistance mechanism	
Required for antimicrobial susceptibility categorization	No
Infection control	Yes
Public health	Yes

3.1 Definition

ESBLs are enzymes that hydrolyze most penicillins and cephalosporins, including oxyimino-β-lactam compounds (cefuroxime, third- and fourth-generation cephalosporins and aztreonam) but not cephamycins or carbapenems. Most ESBLs belong to the Ambler class A of β-lactamases and are inhibited by β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) (1).

3.2 Clinical and/or epidemiological importance

The first ESBL-producing strains were identified in 1983, and since then have been observed worldwide. This distribution is a result of the clonal expansion of producer organisms, the horizontal transfer of ESBL genes on plasmids and, less commonly, their emergence *de novo*. By far the most clinically important groups of ESBLs are CTX-M enzymes, followed by SHV- and TEM-derived ESBLs (2-5). Certain class D OXA-derived enzymes are also included within ESBLs, although inhibition by class A-β-lactamase inhibitors is weaker than for other ESBLs.

ESBL production has been observed mostly in Enterobacteriaceae, first in hospital environments, later in nursing homes, and since around 2000 in the community (outpatients, healthy carriers, sick and healthy animals, food products). The most frequently encountered ESBL-producing species are *Escherichia coli* and *K. pneumoniae.* However, all other clinically-relevant Enterobacteriaceae species are also common ESBL-producers. The prevalence of ESBL-positive isolates depends on a range of factors including species, geographic locality, hospital/ward, group of patients and type of infection, and large variations have been reported in different studies (2,3,6,7). The EARS-Net data for 2011 showed that the rate of invasive *K. pneumoniae* isolates non-susceptible to the third-generation cephalosporins exceeded 10% in the majority of European countries, with some reporting resistance rates higher than 50%. Most of these isolates were presumed to be ESBL-producers based on local ESBL test results (8).

3.3 Mechanisms of resistance

The vast majority of ESBLs are acquired enzymes, encoded by genes on plasmids. The acquired ESBLs are expressed at various levels, and differ significantly in biochemical characteristics such as activity against specific β-lactams (*e.g.* cefotaxime,

ceftazidime, aztreonam). The level of expression and properties of an enzyme, and the co-presence of other resistance mechanisms (other β-lactamases, efflux, altered permeability) result in the large variety of resistance phenotypes observed among ESBL-positive isolates (1-4, 9, 10).

3.4 Recommended methods for detection of ESBLs in Enterobacteriaceae

In many areas, ESBL detection and characterization is recommended or mandatory for infection control purposes. The recommended strategy for the detection of ESBLs in Enterobacteriaceae is based on non-susceptibility to indicator oxyimino-cephalosporins, followed by phenotypic (and in some cases genotypic) confirmation tests (Table 1, Figure 1).

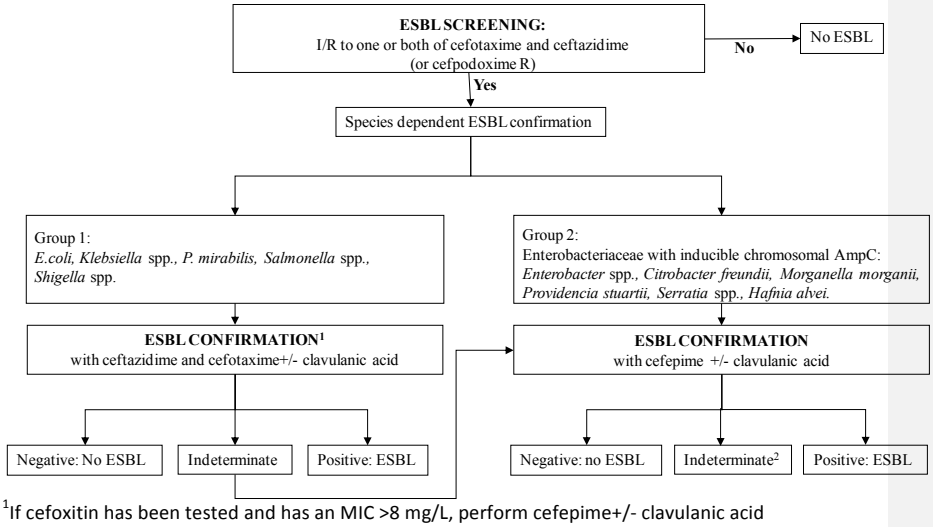
A screening breakpoint of >1mg/L is recommended for cefotaxime, ceftriaxone, ceftazidime, and cefpodoxime, in accordance with the guidelines issued by EUCAST and CLSI (Table 1) (11, 12). The EUCAST clinical breakpoint for Enterobacteriaceae is also S ≤1 mg/L (11). Cefpodoxime is the most sensitive individual indicator cephalosporin for detection of ESBL production and may be used for screening. However, it is less specific than the combination of cefotaxime (or ceftriaxone) and ceftazidime (13, 14) and only the latter compounds are used in the confirmation testing. Corresponding zone diameters for the indicator cephalosporins are shown in Table 1.

Table 1. ESBL screening methods for Enterobacteriaceae (12-18).

Method	**Antibiotic**	**Conduct ESBL-testing if**
Broth or agar dilution[1]	Cefotaxime/ceftriaxone AND Ceftazidime	MIC >1 mg/L for either agent
	Cefpodoxime	MIC >1 mg/L
Disk diffusion[1]	Cefotaxime (5 μg) or	Inhibition zone < 21 mm
	Ceftriaxone (30 μg)	Inhibition zone < 23 mm
	AND Ceftazidime (10 μg)	Inhibition zone < 22 mm
	Cefpdoxime (10 μg)	Inhibition zone < 21 mm

[1] With all methods either test cefotaxime or ceftriaxone AND ceftazidime OR cefpodoxime can be tested alone.

Figure 1. Algorithm for phenotypic detection of ESBLs



[1]If cefoxitin has been tested and has an MIC >8 mg/L, perform cefepime+/- clavulanic acid confirmation test

[2]Cannot be determined as either positive or negative (e.g. if the strip cannot be read due to growth beyond the MIC range of the strip or no clear synergy in combination-disk and double-disk synergy tests). In case confirmation with cefepime +/- clavulanic acid is still indeterminate genotypic testing is required.

3.4.1 ESBL-screening in Enterobacteriaceae

A. Screening in group 1 Enterobacteriaceae (*E. coli*, *Klebsiella* spp., *P. mirabilis*, *Salmonella* spp., *Shigella* spp.)

The recommended methods for ESBL screening in group 1 Enterobacteriaceae are broth dilution, agar dilution, disk diffusion or an automated system (12, 19, 20). It is required that both cefotaxime (or ceftriaxone) and ceftazidime are used as indicator cephalosporins, as there may be large differences in MICs of cefotaxime (or ceftriaxone) and ceftazidime for different ESBL-producing isolates (13, 21, 22).

The algorithm for screening and phenotypic ESBL confirmation methods for group 1 Enterobacteriaceae that are positive in screening tests are described in Figure 1 and Table 2.

B. Screening in group 2 Enterobacteriaceae (*Enterobacter* spp, *Serratia* spp., *Citrobacter freundii*, *Morganella morganii*, *Providencia* spp, *Hafnia alvei*)

For group 2 Enterobacteriaceae it is recommended that ESBL screening is performed according to the methods described above for group 1 *Enterobacteriaceae* (Figure 1 and Table 3) (18). However, a very common mechanism of cephalosporin resistance is derepressed chromosomal AmpC β-lactamase in these species. Since cefepime is stable to AmpC hydrolysis, it can be used in phenotypic testing with clavulanic acid.

3.4.2 Phenotypic confirmation methods

Four of the several phenotypic methods based on the *in vitro* inhibition of ESBL activity by clavulanic acid are recommended for ESBL confirmation, the combination disk test (CDT), the double-disk synergy test (DDST), the ESBL gradient test, and the broth microdilution test (Tables 2 and 3) (19, 20, 23). The CDT showed a better specificity than the ESBL gradient test and with comparable sensitivity in one multi-centre study (24). Manufacturers of automated susceptibility testing systems have implemented detection tests based on the inhibition of ESBL enzymes by clavulanic acid. Performance of confirmation methods differs in different studies, depending on the collection of strains tested and the device used (16-18).

A. Combination disk test (CDT)

For each test, disks or tablets containing the cephalosporin alone (cefotaxime, ceftazidime, cefepime) and in combination with clavulanic acid are applied. The inhibition zone around the cephalosporin disk or tablet combined with clavulanic acid is compared with the zone around the disk or tablet with the cephalosporin alone. The test is positive if the inhibition zone diameter is ≥5 mm larger with clavulanic acid than without (Table 3) (25, 26).

B. Double-disk synergy test (DDST)

Disks containing cephalosporins (cefotaxime, ceftazidime, cefepime) are applied to plates next to a disk with clavulanic acid (amoxicillin-clavulanic acid). A positive result is indicated when the inhibition zones around any of the cephalosporin disks are augmented in the direction of the disk containing clavulanic acid. The distance between the disks is critical and 20mm centre-to-centre has been found to be optimal for cephalosporin 30µg disks; however it may be reduced (15 mm) or expanded (30 mm) for strains with very high or low levels of resistance, respectively (19. The recommendations need to be re-evaluated for disks with lower cephalosporin content, as used in the EUCAST disk diffusion method.

C. Gradient test method

Gradient tests are set up, read and interpreted according to the manufacturer's instructions. The test is positive if ≥8-fold reduction is observed in the MIC of the cephalosporin combined with clavulanic acid compared with the MIC of the cephalosporin alone or if a phantom zone or deformed ellipse is present (see instructions from the manufacturer for illustrations) (Table 3). The test result is indeterminate if the strip cannot be read due to growth beyond the MIC range of the strip. In all other cases the test result is negative. The ESBL gradient test should be used for confirmation of ESBL production only and is not reliable for determination of the MIC.

D. Broth microdilution

Broth microdilution is performed with Mueller-Hinton broth containing serial two-fold dilutions of cefotaxime, ceftazidime and cefepime at concentrations ranging from 0.25 to 512 mg/L, with and without clavulanic acid at a fixed concentration of 4 mg/L. The test is positive if ≥8-fold reduction is observed in the MIC of the

cephalosporin combined with clavulanic acid compared with the MIC of the cephalosporin alone. In all other cases the test result is negative (23).

E. Special considerations in interpretation

ESBL confirmation tests that use cefotaxime as the indicator cephalosporin may be false-positive for *Klebsiella oxytoca* strains with hyperproduction of the chromosomal K1 (OXY-like) β-lactamases (27). A similar phenotype may also be encountered in *Proteus vulgaris, Proteus penneri, Citrobacter koseri* and *Kluyvera* spp. and in some *C. koseri*-related species like *C. sedlakii, C. farmeri* and *C. amalonaticus*, which have chromosomal β-lactamases that are inhibited by clavulanic acid (28, 29). Another possible cause of false-positive results is hyperproduction of SHV-1-, TEM-1- or OXA-1-like broad-spectrum β-lactamases combined with altered permeability (17). Similar problems with false-positive test results for K1-producing *K. oxytoca* may also arise when using confirmation tests based on cefepime only (30).

Table 2. ESBL confirmation methods for Enterobacteriaceae that are positive in the ESBL screening test (see Table 1). Group 1 Enterobacteriaceae (see Figure 1).

Method	Antimicrobial agent (disk content)	ESBL confirmation is positive if
ESBL gradient test	Cefotaxime +/- clavulanic acid	MIC ratio ≥ 8 or deformed ellipse present
	Ceftazidime +/- clavulanic acid	MIC ratio ≥ 8 or deformed ellipse present
Combination disk diffusion test (CDT)	Cefotaxime (30 µg) +/- clavulanic acid (10 µg)	≥ 5 mm increase in inhibition zone
	Ceftazidime (30 µg) +/- clavulanic acid (10 µg)	≥ 5 mm increase in inhibition zone
Broth microdilution	Cefotaxime +/- clavulanic acid (4 mg/L)	MIC ratio ≥ 8
	Ceftazidime +/- clavulanic acid (4 mg/L)	MIC ratio ≥ 8
	Cefepime +/- clavulanic acid (4 mg/L)	MIC ratio ≥ 8
Double disk synergy test (DDST)	Cefotaxime, ceftazidime and cefepime	Expansion of indicator cephalosporin inhibition zone towards amoxicillin-clavulanic acid disk

Table 3. ESBL confirmation methods for Enterobacteriaceae that are positive in the ESBL screening (see Table 1). Group 2 Enterobacteriaceae (see Figure 1).

Method	Antibiotic	Confirmation is positive if
ESBL gradient test Etest ESBL	Cefepime +/- clavulanic acid	MIC ratio ≥ 8 or deformed ellipse present
Combination disk diffusion test	Cefepime (30 µg) +/- clavulanic acid (10 µg)	≥ 5 mm increase in inhibition zone
Broth microdilution	Cefepime +/- clavulanic acid (fixed concentration 4 mg/L)	MIC ratio ≥ 8
Double disk synergy test (DDST)	Cefotaxime, ceftazidime, Cefepime	Expansion of indicator cephalosporin inhibition zone towards amoxicillin-clavulanic acid disk

3.4.3 Phenotypic detection of ESBL in the presence of other β-lactamases that mask synergy

Indeterminate test results (Etest) and false-negative test results (CDT, DDST, Etest and broth microdilution) may result from the high-level expression of AmpC β-lactamases, which mask the presence of ESBLs (19, 31, 32). Isolates with high-level expression of AmpC β-lactamases usually show clear resistance to third-generation cephalosporins. In addition, resistance to cephamycins, e.g. a cefoxitin MIC >8 mg/L, may be indicative of high-level expression of AmpC β-lactamases (31), with the rare exception of ACC β-lactamases, which do not confer cefoxitin resistance (33).

To confirm presence of ESBLs in isolates with high-level expression of AmpC β-lactamases it is recommended that an additional ESBL confirmation test is performed with cefepime as the indicator cephalosporin, as cefepime is usually not hydrolyzed by AmpC β-lactamases. Cefepime may be used in all the CDT, DDST, gradient test or broth dilution test formats (27, 34-36). Alternative approaches include use of cloxacillin, which is a good inhibitor of AmpC enzymes. Test formats are CDT with disks containing the two cephalosporin indicators (cefotaxime and ceftazidime) with both clavulanic acid and cloxacillin together; and standard CDT or DDST on agar plates supplemented with 200-250 mg/L cloxacillin (19). There are also disks or tablets containing both clavulanic acid and cloxacillin on the market, but multicentre evaluations of these products are lacking.

The presence of ESBLs may also be masked by carbapenemases such as MBLs or KPCs (but not OXA-48-like enzymes) and/or severe permeability defects (37, 38). The epidemiological importance of ESBLs in these contexts could be questioned, since the carbapenemase has greater public health importance, but if detection is still considered relevant it is recommended that molecular methods for ESBL detection are used.

It should be remembered that the class D (OXA-type) ESBLs are poorly inhibited by clavulanic acid and therefore cannot be detected by the methods described above (4, 19). These enzymes are currently rare in Enterobacteriaceae.

3.4.4 Genotypic confirmation

For the genotypic confirmation of the presence of ESBL genes, use of PCR and ESBL gene sequencing (3) or a DNA microarray-based method are recommended. Recent evaluations of the Check-KPC ESBL microarray (Check-Points, Wageningen, The Netherlands) with different collections of organisms covering the majority of known ESBL genes showed good performance (39-43). Test results are usually obtained within 24 hours. It should be noted that sporadically occurring ESBL genes and new ESBL genes are not detected by this microarray.

*3.4.5 Quality control*Appropriate strains for quality control of ESBL detection tests are shown in Table 4.

Table 4. Appropriate strains for quality control of ESBL detection tests.

Strain	**Mechanism**
K. pneumoniae ATCC 700603	SHV-18 ESBL
E. coli CCUG62975	CTX-M-1 group ESBL and acquired CMY AmpC
E. coli ATCC 25922	ESBL-negative

3.5 References

1. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for β -lactamases and its correlation with molecular structure. Antimicrob Agents Chemother. 1995;39:211-1233
2. Livermore DM. β-Lactamases in laboratory and clinical resistance. Clin Microbiol Rev. 1995;8:557-584
3. Bradford PA. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev. 2001;14:933-951
4. Naas T, Poirel L, Nordmann P. 2008. Minor extended-spectrum β-lactamases. Clin Microbiol Infect. 2008;14(Suppl1):42-52
5. Cantón R, Novais A, Valverde A, Machado E, Peixe L, Baquero F, Coque TM. Prevalence and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae in Europe. Clin Microbiol Infect. 2008;14(Suppl1):144-153
6. Livermore DM, Cantón R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, Ayala J, Coque TM, Kern-Zdanowicz I, Luzzaro F, Poirel L, Woodford N. CTX-M: changing the face of ESBLs in Europe. J Antimicrob Chemother. 2007;59:165-174
7. Carattoli A. Animal reservoirs for extended-spectrum β-lactamase producers. Clin Microbiol Infect. 2008;14(Suppl1):117-123
8. European Centres for Disease Prevention and Control (ECDC). Antimicrobial resistance surveillance in Europe 2011. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net)
9. Gniadkowski M. 2008. Evolution of extended-spectrum β-lactamases by mutation. Clin Microbiol Infect. 2008;14(Suppl1):11-32

10. Livermore DM. Defining an extended-spectrum β-lactamase. Clin Microbiol Infect. 2008;14(Suppl1):3-10
11. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. EUCAST; 2013. Version 3.0 http://www.eucast.org/clinical_breakpoints/ (last accessed 23 December 2012).
12. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-first Informational Supplement M 100-S21. Wayne, PA, USA: CLSI; 2011.
13. Hope R, Potz NA, Warner M, Fagan EJ, Arnold E, Livermore DM. Efficacy of practised screening methods for detection of cephalosporin-resistant Enterobacteriaceae. J Antimicrob Chemother. 2007;59(1):110-3.
14. Oliver A, Weigel LM, Rasheed JK, McGowan Jr JE Jr, Raney P, Tenover FC. Mechanisms of decreased susceptibility to cefpodoxime in *Escherichia coli*. Antimicrob Agents Chemother. 2002; 46:3829-36.
15. Garrec H, Drieux-Rouzet L, Golmard JL, Jarlier V, Robert J. Comparison of nine phenotypic methods for detection of extended-spectrum beta-lactamase production by Enterobacteriaceae. J Clin Microbiol. 2011;49:1048-57.
16. Leverstein-van Hall MA, Fluit AC, Paauw A, Box AT, Brisse S, Verhoef J. Evaluation of the Etest ESBL and the BD Phoenix, VITEK 1, and VITEK 2 automated instruments for detection of extended-spectrum β-lactamases in multiresistant *Escherichia coli* and *Klebsiella* spp. J Clin Microbiol. 2002;40:3703-11.
17. Spanu T, Sanguinetti M, Tumbarello M, D'Inzeo T, Fiori B, Posteraro B, Santangelo R, Cauda R, Fadda G. Evaluation of the new VITEK 2 extended-spectrum β-lactamase (ESBL) test for rapid detection of ESBL production in Enterobacteriaceae isolates. J Clin Microbiol. 2006;44:3257-62.
18. Thomson KS, Cornish NE, Hong SG, Hemrick K, Herdt C, Moland ES. Comparison of Phoenix and VITEK 2 extended-spectrum-β-lactamase detection tests for analysis of *Escherichia coli* and *Klebsiella* isolates with well-characterized β-lactamases. J Clin Microbiol. 2007;45:2380-4.
19. Drieux L, Brossier F, Sougakoff W, Jarlier V. Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae: review and bench guide. Clin Microbiol Infect. 2008; 14 (Suppl 1):90-103.
20. Paterson DL, Bonomo RA. Extended-spectrum β-lactamases: a clinical update. Clin Microbiol Rev. 2005;18:657-86
21. Biedenbach DJ, Toleman M, Walsh TR, Jones RN. Analysis of *Salmonella* spp. with resistance to extended-spectrum cephalosporins and fluoroquinolones isolated in North America and Latin America: report from the SENTRY Antimicrobial Surveillance Program (1997-2004). Diagn Microbiol Infect Dis. 2006;54:13-21.
22. Hirakata Y, Matsuda J, Miyazaki Y, Kamihira S, Kawakami S et al. Regional variation in the prevalence of extended-spectrum β-lactamase-producing clinical isolates in the Asia-Pacific region (SENTRY 1998-2002). Diagn Microbiol Infect Dis. 2005;52:323-9.
23. Jeong SH, Song W, Kim JS, Kim HS, Lee KM. Broth microdilution method to detect extended-spectrum β-lactamases and AmpC β-lactamases in Enterobacteriaceae isolates by use of clavulanic acid and boronic acid as inhibitors. J Clin Microbiol. 2009;47:3409-12.
24. Platteel TN, Cohen Stuart JW, de Neeling AJ, Voets GM, Scharringa J et al. Multi-centre evaluation of a phenotypic extended spectrum β-lactamase detection guideline in the routine setting. Clin Microbiol Infect. 2013;19:70-6.
25. M'Zali FH, Chanawong A, Kerr KG, Birkenhead D, Hawkey PM. Detection of extended-spectrum β-lactamases in members of the family Enterobacteriaceae: comparison of the MAST DD test, the double disc and the Etest ESBL. J Antimicrob Chemother. 2000;45:881-5.
26. Towne TG, Lewis JS 2nd, Herrera M, Wickes B, Jorgensen JH. Detection of SHV-type extended-spectrum β-lactamase in *Enterobacter* isolates. J Clin Microbiol. 2010;48:298-9.
27. Stürenburg E, Sobottka I, Noor D, Laufs R, Mack D. Evaluation of a new cefepime-clavulanate ESBL Etest to detect extended-spectrum β-lactamases in an Enterobacteriaceae strain collection. J Antimicrob Chemother. 2004;54:134-8.
28. Nukaga M, Mayama K, Crichlow GV, Knox JR. Structure of an extended-spectrum class A β-lactamase from *Proteus vulgaris* K1. J Mol Biol. 2002;317:109-17.

29. Petrella S, Renard M, Ziental-Gelus N, Clermont D, Jarlier V, Sougakoff W. Characterization of the chromosomal class A β-lactamase CKO from *Citrobacter koseri*. FEMS Microbiol Lett. 2006;254:285-92.
30. Stürenburg E, Sobottka I, Noor D, Laufs R, Mack D. Evaluation of a new cefepime-clavulanate ESBL Etest to detect extended-spectrum beta-lactamases in an Enterobacteriaceae strain collection. J Antimicrob Chemother. 2004; 54:134-8.
31. Jacoby GA. AmpC β-lactamases. Clin Microbiol Rev. 2009;22:161-82
32. Munier GK, Johnson CL, Snyder JW, Moland ES, Hanson ND, Thomson KS. Positive extended-spectrum-β-lactamase (ESBL) screening results may be due to AmpC β-lactamases more often than to ESBLs. J Clin Microbiol. 2010;48:673-4.
33. Bauernfeind A, Schneider I, Jungwirth R, Sahly H, Ullmann U. A novel type of AmpC β-lactamase, ACC-1, produced by a *Klebsiella pneumoniae* strain causing nosocomial pneumonia. Antimicrob Agents Chemother. 1999;43:1924-31.
34. Polsfuss S, Bloemberg GV, Giger J, Meyer V, Böttger EC, Hombach M. Evaluation of a diagnostic flow chart for detection and confirmation of extended spectrum β-lactamases (ESBL) in Enterobacteriaceae. Clin Microbiol Infect. 2012;18:1194-204.
35. Yang JL, Wang JT, Lauderdale TL, Chang SC. Prevalence of extended-spectrum beta-lactamases in Enterobacter cloacae in Taiwan and comparison of 3 phenotypic confirmatory methods for detecting extended-spectrum beta-lactamase production. J Microbiol Immunol Infect. 2009;42:310-6.
36. Jeong SH, Song W, Kim JS, Kim HS, Lee KM. Broth microdilution method to detect extended-spectrum beta-lactamases and AmpC beta-lactamases in enterobacteriaceae isolates by use of clavulanic acid and boronic acid as inhibitors. J Clin Microbiol. 2009;47:3409-12.
37. Tsakris A, Poulou A, Themeli-Digalaki K, Voulgari E, Pittaras T, Sofianou D, Pournaras S, Petropoulou D. Use of boronic acid disk tests to detect extended- spectrum β-lactamases in clinical isolates of KPC carbapenemase-possessing Enterobacteriaceae. J Clin Microbiol. 2009;47:3420-6.
38. March A, Aschbacher R, Dhanji H, Livermore DM, Böttcher A et al. Colonization of residents and staff of a long-term-care facility and adjacent acute-care hospital geriatric unit by multiresistant bacteria. Clin Microbiol Infect. 2010;16:934-44.
39. Cohen Stuart J, Dierikx C, Al Naiemi N, Karczmarek A, Van Hoek AH et al. Rapid detection of TEM, SHV and CTX-M extended-spectrum β-lactamases in Enterobacteriaceae using ligation-mediated amplification with microarray analysis. J Antimicrob Chemother. 2010;65:1377-81
40. Endimiani A, Hujer AM, Hujer KM, Gatta JA, Schriver AC et al. Evaluation of a commercial microarray system for detection of SHV-, TEM-, CTX-M-, and KPC-type β-lactamase genes in Gram-negative isolates. J Clin Microbiol. 2010;48:2618-22.
41. Naas T, Cuzon G, Truong H, Bernabeu S, Nordmann P. Evaluation of a DNA microarray, the Check-Points ESBL/KPC array, for rapid detection of TEM, SHV, and CTX-M extended-spectrum β-lactamases and KPC carbapenemases. Antimicrob Agents Chemother. 2010;54:3086-92.
42. Platteel TN, Stuart JW, Voets GM, Scharringa J, van de Sande N et al. Evaluation of a commercial microarray as a confirmation test for the presence of extended-spectrum β-lactamases in isolates from the routine clinical setting. Clin Microbiol Infect. 2011;17:1435-8.
43. Willemsen I, Overdevest I, Al Naiemi N, Rijnsburger M, Savelkoul P et al. New diagnostic microarray (Check-KPC ESBL) for detection and identification of extended-spectrum β-lactamases in highly resistant Enterobacteriaceae. J Clin Microbiol. 2011;49:2985-7.

4. Acquired AmpC β-lactamase-producing Enterobacteriaceae

Importance of detection of resistance mechanism	
Required for antimicrobial susceptibility categorization	No
Infection control	Yes
Public health	Yes

4.1 Definition

AmpC-type cephalosporinases are Ambler class C β-lactamases. They hydrolyze penicillins, cephalosporins (including the third-generation but usually not the fourth-generation compounds) and monobactams. In general, AmpC-type enzymes are poorly inhibited by the classical ESBL inhibitors, especially clavulanic acid (1).

4.2 Clinical and/or epidemiological importance

The first isolates producing acquired AmpCs were identified at the end of 1980s, and since then they have been observed globally as a result of clonal spread and horizontal transfer of AmpC genes (often referred to as plasmid-mediated AmpC). There are several lineages of mobile AmpC genes, originating from natural producers, namely the *Enterobacter* group (MIR, ACT), the *C. freundii* group (CMY-2-like, LAT, CFE), the *M. morganii* group (DHA), the *Hafnia alvei* group (ACC), the *Aeromonas* group (CMY-1-like, FOX, MOX) and the *Acinetobacter baumannii* group (ABA). The most prevalent and most widely disseminated are the CMY-2-like enzymes, although the inducible DHA-like β-lactamases and some others have also spread extensively (1).

The major producer species of acquired AmpCs are *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Salmonella enterica* and *P. mirabilis*. Isolates with these enzymes have been recovered from both hospitalized and community patients, and they were recognized earlier than classical ESBL-enzymes in farm animals and in food products (in *E. coli* and *S. enterica*). Although the acquired AmpCs have been spread widely and been recorded in multi-centre studies of enterobacterial resistance to third-generation cephalosporins, their overall frequency has remained far below that of ESBLs. However, in some local and specific epidemiological settings, the significance of organisms producing these enzymes may substantially increase (1-5).

4.3 Mechanisms of resistance

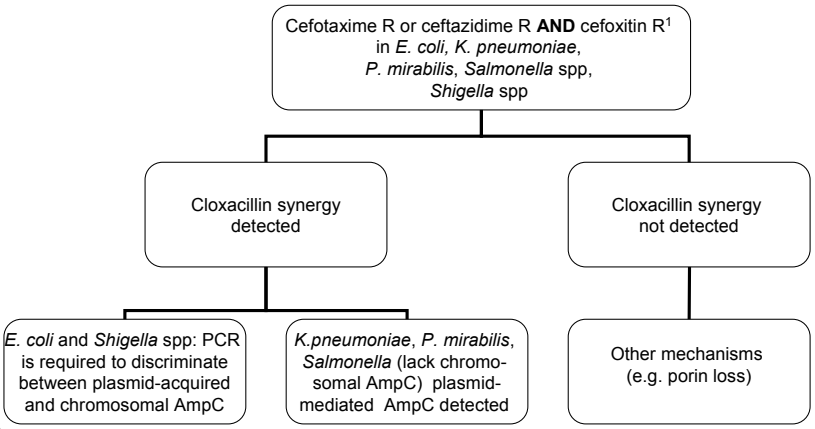
Numerous Enterobacteriaceae and other some other Gram-negative bacilli produce natural AmpCs, either constitutively at a trace level (*e.g. E. coli, Acinetobacter baumannii*) or inducibly (*e.g. Enterobacter* spp., *C. freundii*, *M. morganii*, *P. aeruginosa*). The derepression or hyperproduction of natural AmpCs are due to various genetic changes and confer high-level resistance to cephalosporins and to penicillin-β-lactamase inhibitor combinations. The class C cephalosporinases can also

occur as acquired enzymes, mainly in Enterobacteriaceae. Except for a few inducible types (e.g. DHA), the acquired AmpCs are expressed constitutively, conferring resistance similar to that in the derepressed or hyperproducing mutants of natural AmpC producers. Resistance levels depend on the amounts of enzymes expressed, as well as the presence of other resistance mechanisms. Similar to ESBLs, the acquired AmpCs are usually encoded by plasmid-mediated genes (1-3).

4.4 Recommended methods for detection of acquired AmpC in Enterobacteriaceae

A cefoxitin MIC >8 mg/L combined with a ceftazidime and/or cefotaxime MIC >1mg/L may be used as phenotypic criteria for investigation of AmpC production in group 1 Enterobacteriaceae, although this strategy will not detect ACC-1, a plasmid-mediated AmpC that does not hydrolyze cefoxitin (6). It should be noted that cefoxitin resistance may also be due to porin deficiency (1).

Figure 1. Algorithm for AmpC detection.



[1]Cefoxitin R is here defined as non-wild type (MIC >8 mg/L or zone diameter <19 mm). Investigation of isolates with non-susceptibility to cefotaxime and ceftazidime is an approach with higher sensitivity but lower specificity compared with focusing on cefoxitin resistant isolates (7). AmpC can also be present in isolates with a positive ESBL-test (clavulanic acid synergy). It could therefore be relevant to carry out testing regardless of the result of the ESBL test. For laboratories not testing cefoxitin, susceptible to cefepime together with resistant to cefotaxime and/or ceftazidime is another phenotypic indicator of AmpC, although less specific.

Phenotypic AmpC confirmation tests are generally based on inhibition of AmpC by either cloxacillin or boronic acid derivatives. However, boronic acid derivatives also inhibit class A carbapenemases. Although data evaluating these methods is sparse, reasonably accurate detection with in-house methods has been described (8-10) as well as with commercially available tests such as the Mast "AmpC Detection Disc Set" (sensitivity 96-100%, specificity 98%-100%) (11, 12), the AmpC gradient test,

currently available only from bioMérieux (sensitivity 84-93%, specificity 70-100%) (12, 13) and Rosco tablets with cefotaxime-cloxacillin and ceftazidime-cloxacillin (sensitivity 96%, specificity 92%) (7,14). For *E. coli* however, AmpC confirmation tests cannot discriminate between acquired AmpC and constitutive hyperproduction of the chromosomal AmpC.

The presence of acquired AmpCs may also be confirmed using PCR-based methods (15, 16), or with a DNA microarray-based method (Check-Points) (17).

Appropriate strains for quality control of AmpC detection tests are shown in Table 1.

Table 1. Appropriate strains for quality control of AmpC detection tests.

Strain	Mechanism
E. coli CCUG 58543	Acquired CMY-2 AmpC
E. coli CCUG62975	Acquired CMY AmpC and CTX-M-1 group ESBL
E. coli ATCC 25922	AmpC negative.

4.5 References

1. Jacoby GA. AmpC beta-lactamases. Clin Microbiol Rev. 2009;22:161-82
2. Philippon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type β-lactamases. Antimicrob Agents Chemother. 2002;46:1-11
3. Beceiro A, Bou G. Class C β-lactamases: an increasing problem worldwide. Rev Med Microbiol. 2004;15:141-152
4. Empel J, Hrabák J, Kozińska A, Bergerová T, Urbášková P, Kern-Zdanowicz I, Gniadkowski M. DHA-1-producing *Klebsiella pneumoniae* in a teaching hospital in the Czech Republic. Microb Drug Resist. 2010;16:291-295
5. D'Andrea MM, Literacka E, Zioga A, Giani T, Baraniak A, Fiett J, Sadowy E, Tassios PT, Rossolini GM, Gniadkowski M, Miriagou V. Evolution of a multi-drug-resistant *Proteus mirabilis* clone with chromosomal AmpC-type cephalosporinases spreading in Europe. Antimicrob Agents Chemother. 2011;55:2735-2742
6. Bauernfeind A, Schneider I, Jungwirth R, Sahly H, Ullmann U. A novel type of AmpC β-lactamase, ACC-1, produced by a *Klebsiella pneumoniae* strain causing nosocomial pneumonia. Antimicrob Agents Chemother. 1999;43:1924-31.
7. Edquist P, Ringman M, Liljequist BO, Wisell KT, Giske CG. Phenotypic detection of plasmid-acquired AmpC in Escherichia coli-evaluation of screening criteria and performance of two commercial methods for the phenotypic confirmation of AmpC production. Eur J Clin Microbiol Infect Dis. 2013; 32:1205-10
8. Yagi T, Wachino J, Kurokawa H, Suzuki S, Yamane K et al. Practical methods using boronic acid compounds for identification of class C beta-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*. J Clin Microbiol. 2005;43:2551-8.
9. Tenover FC, Emery SL, Spiegel CA, Bradford PA, Eells S et al. Identification of plasmid-mediated AmpC β-lactamases in *Escherichia coli*, *Klebsiella* spp., and *Proteus* spp. can potentially improve reporting of cephalosporin susceptibility testing results. J Clin Microbiol. 2009;47:294-9.
10. Tan TY, Ng LS, He J, Koh TH, Hsu LY. Evaluation of screening methods to detect plasmid-mediated AmpC in *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. Antimicrob Agents Chemother. 2009;53:146-9

11. Halstead FD, Vanstone GL, Balakrishnan I. An evaluation of the Mast D69C AmpC Detection Disc Set for the detection of inducible and derepressed AmpC β-lactamases. J Antimicrob Chemother. 2012;67:2303-4.
12. Ingram PR, Inglis TJ, Vanzetti TR, Henderson BA, Harnett GB, Murray RJ. Comparison of methods for AmpC β-lactamase detection in Enterobacteriaceae. J Med Microbiol. 2011; 60(Pt 6):715-21.
13. Peter-Getzlaff S, Polsfuss S, Poledica M, Hombach M, Giger J et al. Detection of AmpC β-lactamase in *Escherichia coli*: comparison of three phenotypic confirmation assays and genetic analysis. J Clin Microbiol. 2011;49:2924-32.
14. Hansen F, Hammerum AM, Skov RL, Giske CG, Sundsfjord A, Samuelsen O. Evaluation of ROSCO Neo-Sensitabs for phenotypic detection and subgrouping of ESBL-, AmpC- and carbapenemase-producing Enterobacteriaceae. APMIS. 2012;120:724-32.
15. Pérez-Pérez FJ, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol. 2002;40:2153-62.
16. Brolund A, Wisell KT, Edquist PJ, Elfström L, Walder M, Giske CG. Development of a real-time SYBRGreen PCR assay for rapid detection of acquired AmpC in Enterobacteriaceae. J Microbiol Methods. 2010; 82:229-33.
17. Cuzon G, Naas T, Bogaerts P, Glupczynski Y, Nordmann P. Evaluation of a DNA microarray for the rapid detection of extended-spectrum β-lactamases (TEM, SHV and CTX-M), plasmid-mediated cephalosporinases (CMY-2-like, DHA, FOX, ACC-1, ACT/MIR and CMY-1-like/MOX) and carbapenemases (KPC, OXA-48, VIM, IMP and NDM). J Antimicrob Chemother. 2012;67:1865-9.

5. Methicillin resistant *Staphylococcus aureus* (MRSA)

Importance of detection of resistance	
Required for antimicrobial susceptibility categorization	Yes
Infection control	Yes
Public health	Yes

5.1 Definition

S. aureus isolates with an auxiliary penicillin-binding protein (PBP2a or the recently discovered alternative PBP2 encoded by *mecC*) for which β-lactam agents, except for the novel class of cephalosporins having anti-MRSA activity, have low affinity.

5.2 Clinical and/or epidemiological importance

Methicillin resistant *S. aureus* is a major cause of morbidity and mortality worldwide (1,2). The mortality of MRSA bloodstream infections is doublethat of similar infections caused by methicillin susceptible strains due to delayed adequate treatment and inferior alternative treatment regimens (3). MRSA infections are endemic in both hospitals and the community in all parts of the world.

5.3 Mechanisms of resistance

The main mechanism of resistance is production of an auxiliary penicillin-binding protein, PBP2a or the recently discovered alternative PBP2 encoded by *mecC*, which render the isolate resistant to all β-lactams except for the novel class of cephalosporins, which have sufficiently high affinity to PBP2a, and probably also the PBP encoded by *mecC*, to be active against MRSA (4). The auxiliary PBPs are encoded by the *mecA* gene or the recently described *mecC* (formerly known as $mecA_{LGA251}$) (5) respectively. The *mec* element is foreign to *S. aureus* and is not present in methicillin susceptible *S. aureus.* Strains with marked heterogeneous expression of the *mecA* gene and frequently low MICs of oxacillin hamper the accuracy of susceptibility testing (5). Furthermore, some isolates express low-level resistance to oxacillin, but are *mecA* and *mecC* negative and do not produce alternative PBPs [borderline susceptible *S. aureus* (BORSA)]. These strains are relatively rare and the mechanism of resistance is poorly characterized, but may include hyperproduction of β-lactamases or alteration of the pre-existing PBPs (6).

5.4 Recommended methods for detection of methicillin resistance in *S. aureus*

Methicillin/oxacillin resistance can be detected both phenotypically by MIC determination, disk diffusion tests or latex agglutination to detect PBP2a, and genotypically using PCR.

5.4.1 Detection by MIC determination or disk diffusion

The heterogeneous expression of resistance particularly affects MICs of oxacillin. Cefoxitin is a very sensitive and specific marker of *mecA/mecC*-mediated methicillin

resistance and is the agent of choice for disk diffusion. Disk diffusion using oxacillin is discouraged and interpretive zone diameters are no longer included in the EUCAST breakpoint table due to poor correlation with the presence of *mecA*. Strains with increased MICs of oxacillin (MIC >2 mg/L), but which remain susceptible to cefoxitin (zone diameter ≥22 mm, MIC ≤4 mg/L) are uncommon. If oxacillin is tested and gives a different interpretation than with cefoxitin the interpretation should be as shown below. It is recommended to subject such strains to phenotypic or genotypic investigations for *mecA* or *mecC*.

Table 1. Interpretation when oxacillin and cefoxitin results are discrepant.

		Cefoxitin result by MIC or disk diffusion	
		S	R
Oxacillin result by MIC	S	Report as oxacillin S	Report as oxacillin R
	R	Report as oxacillin R	Report as oxacillin R

A. Broth microdilution:
Standard methodology (ISO 20776-1) is used and strains with cefoxitin MICs >4 mg/L should be reported as methicillin resistant.

B. Disk diffusion**:** The EUCAST disk diffusion method is used. Strains with a cefoxitin (30 µg disk) zone diameter <22 mm should be reported as methicillin resistant.

5.4.2 Detection with genotypic and latex agglutination methods

Genotypic detection of the *mecA* gene by PCR and detection of the PBP2a protein with latex agglutination kits is possible using commercial or in-house assays. However, *mecC* and the PBP encoded by this gene can at present not be detected using commercially available genotypic or phenotypic methods. Primers and methods for detection of *mecC* have recently been published (7, 8).

5.4.3 Control strains

Appropriate strains for quality control of methicillin susceptibility tests are shown in Table 2.

Table 2. Appropriate strains for quality control of methicillin susceptibility tests.

Strain	**Mechanism**
S. aureus ATCC 29213	Methicillin susceptible
S. aureus NCTC 12493	Methicillin resistant (*mecA*)
S. aureus NCTC 13552	Methicillin resistant (*mecC*)

5.5 References

1. Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, Carmeli Y. Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis. Clin Infect Dis. 2003;36:53-9.
2. de Kraker ME, Wolkewitz M, Davey PG, Koller W, Berger J, et al. Clinical impact of antimicrobial resistance in European hospitals: excess mortality and length of hospital stay related to methicillin-resistant *Staphylococcus aureus* bloodstream infections. Antimicrob Agents Chemother. 2011;55:1598-605.
3. de Kraker ME, Davey PG, Grundmann H; BURDEN study group. Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteremia: estimating the burden of antibiotic resistance in Europe. PLoS Med. 2011;8(10):e1001104.
4. Chambers HF, Deleo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol. 2009;7:629-41.
5. García-Álvarez L, Holden MT, Lindsay H, Webb CR, Brown DF, et al. Meticillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect Dis. 2011;11:595-603
6. Chambers HF. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clin Microbiol Rev. 1997;10:781-91.
7. Stegger M, Andersen PS, Kearns A, Pichon B, Holmes MA, Edwards G, Laurent F, Teale C, Skov R, Larsen AR. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mecA* or the new *mecA* homologue $mecA_{LGA251}$. Clin Microbiol Infect. 2012; 4:395-400.
8. Pichon B, Hill R, Laurent F, Larsen AR, Skov RL, Holmes M, Edwards GF, Teale C, Kearns AM. Development of a real-time quadruplex PCR assay for simultaneous detection of *nuc*, Panton-Valentine leucocidin (PVL), *mecA* and homologue $mecA_{LGA251}$. J Antimicrob Chemother. 2012; 67:2338-41.

6. Glycopeptide non-susceptible *Staphylococcus aureus*

Importance of detection of resistance	
Required for antimicrobial susceptibility categorization	Yes
Infection control	Yes
Public health	Yes

6.1 Definition

The EUCAST clinical MIC breakpoint for resistance to vancomycin in *S. aureus* is >2 mg/L. In recent years vancomycin breakpoints have been lowered, thereby removing the former intermediate group. However, there are important differences in the mechanism of resistance in VanA-mediated high-level glycopeptide resistant *S. aureus* (GRSA) and non-VanA mediated low-level resistant isolates. Hence, the terms glycopeptide intermediate *S. aureus* (GISA) and heteroresistant glycopeptide intermediate *S. aureus* (hGISA) have been maintained for isolates with non-VanA-mediated low-level resistance to vancomycin. The MIC should always be determined when using vancomycin to treat a patient with severe *S. aureus* infection. In selected cases, e.g. when therapeutic failure is suspected, testing for hGISA may also be warranted. Due to the complexity of confirming hGISA, antimicrobial surveillance is focused on detection of GISA and GRSA.

GRSA: Glycopeptide resistant *S. aureus*:
S. aureus isolates with high-level resistance to vancomycin (MIC >8 mg/L).

GISA: glycopeptide intermediate *S. aureus*
S. aureus isolates with low-level resistance to vancomycin (MIC 4 - 8 mg/L).

hGISA: Heterogeneous glycopeptide intermediate *S. aureus.*
S. aureus isolates susceptible to vancomycin (MICs ≤2mg/L) but with minority populations (1 in 10^6 cells) with vancomycin MIC >2 mg/L, as judged by population analysis profile investigation.

6.2 Clinical and/or epidemiological importance

There are no recent investigations of the prevalence of isolates with reduced susceptibility to glycopeptides in Europe. Based on reports from single institutions it is estimated that the prevalence of hGISA is ≤2% of MRSA in Europe, with GISA below 0.1% (1). GRSA has not yet been reported in Europe and is currently extremely rare worldwide (1). The prevalence of hGISA may be considerably higher locally (1), most often associated with spread of specific clonal lineages (2). Almost all isolates with elevated MIC (GISA) or containing resistant subpopulations (hGISA) are MRSA.

The clinical significance of hGISA has been difficult to determine as no well-controlled prospective studies have been performed. However, presence of the hGISA phenotype is believed to be associated with poorer outcome, at least in

serious infections (1, 2). It is therefore prudent to investigate for hGISA in bloodstream infections not responding to therapy. Recently there has been increasing evidence that isolates with MICs in the upper part of the susceptible range (MIC >1 mg/L) are associated with poorer outcome and may be linked to increased mortality, at least in bloodstream infections (2-7). It is still uncertain whether the presence of resistant subpopulations is responsible for the poorer outcome, as it could also be a consequence of the slightly elevated vancomycin MICs observed for these strains.

The mechanism of hGISA is complex and detection relies on population analysis (8), which is cumbersome, requires special equipment and needs a high level of technical expertise. Methodology for detection of hGISA will be outlined, but for surveillance purposes reporting is restricted to GISA and GRSA, which are together defined as isolates with an MIC >2mg/L.

6.3 Mechanism of resistance

For GRSA the resistance is mediated by the *vanA* gene exogenously acquired from enterococci. For both GISA and hGISA isolates the resistance is endogenous (i.e. chromosomal mutations) and the mechanism highly complex, with no single gene being responsible. The GISA/hGISA phenotype is linked to a thickening of the bacterial cell wall, with hyperproduction of glycopeptide binding targets. The hGISA phenotype is often unstable in the laboratory, but hGISA have the capacity to develop into GISA *in vivo* (1).

6.4 Recommended methods for detection of glycopeptide non-susceptible *S. aureus*

Disk diffusion CANNOT be used to test for either hGISA or GISA, but can be used to test for GRSA.

6.4.1 MIC determination

Broth microdilution methodology as recommended by EUCAST (ISO 20776-1) is the gold standard, but MICs may also be determined by gradient strip methods, agar dilution or automated systems. It should be noted that the results with gradient strip methods may be 0.5-1 two-fold dilution steps higher than the results obtained by broth microdilution (7). The EUCAST breakpoint for resistance to vancomycin in *S. aureus* is MIC >2 mg/L. Isolates with confirmed MICs >2 mg/L (according to broth microdilution) should be referred to a reference laboratory. hGISA are not detected by MIC determination.

6.4.2 Test detecting GRSA, GISA and hGISA

Detection of hGISA has proven difficult and detection is therefore divided into screening and confirmation. For screening, a number of specialised methods have been developed. Confirmation is by analysing the population profile of the isolate on

agar plates containing a range of vancomycin concentrations (PAP-AUC) (8). This method is technically challenging without extensive experience and consequently is mostly performed by reference laboratories. A method based on a vancomycin and casein screening agar (9) has shown high sensitivity and specificity, but has so far only been evaluated in one study, and for that reason not been included. The following methods will all detect GRSA and GISA, and have been evaluated in a multicentre study (10).

A. Macro gradient test:
This test gives an indication of reduced vancomycin susceptibility but note that the readings are not MICs. Furthermore, the test does not differentiate between hGISA, GISA and GRSA. The test is set up according to the manufacturer's instructions. Note also that the inoculum is higher (2,0 McFarland) than with standard gradient tests. A positive result is indicated by readings ≥8mg/L for both vancomycin and teicoplanin, OR ≥ 12mg/L for teicoplanin alone.

As both criteria include teicoplanin, testing of vancomycin could be dependent on the result of the teicoplanin test. The algorithm would then be:

- Teicoplanin reading ≥12 mg/L: GRSA, GISA or hGISA
- Teicoplanin reading 8 mg/L: Test vancomycin. If vancomycin reading is ≥8 mg/L then GRSA, GISA or hGISA
- Teicoplanin reading <8 mg/L: Not GRSA, GISA or hGISA

B. Glycopeptide resistance detection (GRD) gradient test:
Test according to the manufacturer's instructions. The test is considered positive if the GRD strip result is ≥8 mg/L for either vancomycin or teicoplanin.

C. Teicoplanin screening agar:
A Mueller Hinton plate containing 5 mg/L teicoplanin is used (10). Several colonies are suspended in 0.9% saline to obtain an inoculum with equivalent turbidity to a 2.0 McFarland standard. Ten microliters of inoculum is delivered as a spot on the surface of the agar, and the plate incubated at 35°C in air for 24 to 48 h. Growth of more than two colonies at 48h indicates suspected reduced susceptibility to glycopeptides.

D. Confirmatory testing for hGISA/GISA:
Any isolate screening positive for reduced susceptibility and not identified as GRSA or GISA by MIC determination may be hGISA and may be investigated by population analysis profile-area under curve (PAP-AUC) (8), typically by referral to a reference laboratory.

6.4.3 Control strains

Appropriate strains for quality control of glycopeptide susceptibility tests are shown in table 2.

Table 1. Appropriate strains for quality control of glycopeptide susceptibility tests.

Strain	Mechanism
S. aureus ATCC 29213	Glycopeptide susceptible
S. aureus ATCC 700698	hGISA (Mu3)
S. aureus ATCC 700699	GISA (Mu50)

6.5 References

1. Howden BP, Davies JK, Johnson PDR, Stinear TP, Grayson ML. Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection and clinical implications. Clin Microbiol Rev 2010;1: 99-139
2. Van Hal SJ, Lodise TP, Paterson DL. The clinical significance of vancomycin minimum inhibitory concentration in *Staphylococcus aureus* infections: a systematic review and meta-analysis. Clinical Infectious Diseases 2012; 54: 755-771.
3. Chang HJ, Hsu PC, Yang CC, Siu LK, Kuo AJ, et al. Influence of teicoplanin MICs on treatment outcomes among patients with teicoplanin-treated methicillin resistant *Staphylococcus aureus* bacteraemia: a hospital based retrospective study. J. Antimicrob Chemother 2012, 67:736-41.
4. Honda H, Doern CD, Michael-Dunne W Jr, Warren DK. The impact of vancomycin susceptibility on treatment outcomes among patients with methicillin resistant *Staphylococcus aureus* bacteremia. BMC Infect Dis. 2011; 5:11:335.
5. Lodise TP, Graves J, Evans A, Graffunder E, Helmecke M, et al. Relationship between vancomycin MIC and failure among patients with methicillin-resistant *Staphylococcus aureus* bacteremia treated with vancomycin. Antimicrob Agents Chemother 2008; 52*:* 3315-20
6. Rojas L, Bunsow E, Munoz P, Cercenado E, Rodrigueuz-Creixems, Bouza E. Vancomycin MICs do not predict the outcome of methicillin-resistant *Staphylococcus aureus* bloodstream infections in correctly treated patients. J. Antimicrob Chemother 2012; 7: 1760-8.
7. Sader HS, Jones RN, Rossi KL, Rybak MJ. Occurrence of vancomycin tolerant and heterogeneous vancomycin resistant strains (hVISA) among *Staphylococcus aureus* causing bloodstream infections in nine USA hospitals. Antimicrob Chemother. 2009; 64: 1024-8.
8. Wootton M, Howe RA, Hillman R, Walsh TR, Bennett PM, MacGowan AP. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in *Staphylococcus aureus* in a UK hospital. J Antimicrob Chemother. 2001; 47: 399-403
9. Satola SW, Farley MM, Anderson KF, Patel JB. Comparison of Detection Methods for Heteroresistant Vancomycin-Intermediate *Staphylococcus aureus*, with the Population Analysis Profile Method as the Reference Method. J Clin Microbiol. 2011; 49: 177–183.
10. Wootton M, MacGowan AP, Walsh TR, Howe RA. A multicenter study evaluating the current strategies for isolating *Staphylococcus aureus* strains with reduced susceptibility to glycopeptides. J Clin Microbiol. 2007;45:329-32.

7. Vancomycin resistant *Enterococcus faecium* and *Enterococcus faecalis*

Importance of detection of resistance	
Required for antimicrobial susceptibility categorization	Yes
Infection control/public health	Yes
Public health	Yes

7.1 Definition

Enterococcus faecium or *Enterococcus faecalis* with resistance to vancomycin (VRE) (vancomycin MIC >4 mg/L).

7.2 Clinical and/or epidemiological importance

Enterococci, especially *E. faecium*, are generally resistant to most clinically available antimicrobial agents. Treatment of infections caused by vancomycin resistant enterococci (VRE) is therefore difficult, with few treatment options. VRE are known to spread efficiently and persist in the hospital environment, and can colonize many individuals of which only a few may develop enterococcal infections (6, 7). Isolates harbouring VanB are usually phenotypically susceptible to teicoplanin. There are two case reports of selection of teicoplanin resistance during treatment of enterococci harbouring VanB (8, 9), but reports of clinical failures are lacking and the current EUCAST recommendation is to report the result for teicoplanin as found. Typical MIC values for the clinically most important Van enzymes are shown in Table 1.

Table 1. Typical MICs of glycopeptides for enterococci harbouring VanA or VanB.

Glycopeptide	MIC (mg/L)	
	VanA	VanB
Vancomycin	64-1024	4-1024
Teicoplanin	8-512	0.06-1

7.3 Mechanism of resistance

Clinically-relevant resistance is most often mediated by plasmid-encoded VanA and VanB ligases that replace the terminal D-Ala in the peptidoglycan with D-Lac. This substitution reduces the binding of glycopeptides to the target. VanA strains exhibit resistance to both vancomycin and teicoplanin, whereas VanB strains usually remain susceptible to teicoplanin due to lack of induction of the resistance operon. Other Van enzymes of lower prevalence are VanD, VanE, VanG, VanL, VanM and VanN (1-4).

Additional enterococcal species (i.e. *E. raffinosus, E. gallinarum* and *E. casseliflavus)*, may contain *vanA, vanB* or other *van* genes encoding enzymes listed above, but these strains are relatively rare. Chromosomally-encoded VanC enzymes are found in all *E. gallinarum* and *E. casseliflavus* isolates*.* VanC mediates low-level vancomycin resistance (MIC 4-16 mg/L) but should generally not be considered important from an infection control point of view (5).

7.4 Recommended methods for detection of glycopeptide resistance in *E. faecium* and *E. faecalis*

Vancomycin resistance can be detected by MIC determination, disk diffusion and the breakpoint agar method. For all three methods it is essential that plates are incubated for a full 24 h in order to detect isolates with inducible resistance.

All three methods readily detect *vanA*-mediated resistance. Detection of *vanB*-mediated resistance is more challenging. MIC determination by agar or broth dilution is accurate, but is seldom used in routine laboratories (10, 11). Older reports show that detection of *vanB*-mediated resistance is problematic for automated methods (12). Since then updates have been made to the automated methods, but more recent studies on whether the performances of these methods for detection of *vanB*-mediated resistance have improved are lacking. Disk diffusion with a 5µg vancomycin disk can be difficult but the test performs well provided the guidelines for reading as specified by EUCAST are followed meticulously (unpublished data from the EUCAST reference laboratory).

When interpreting the MIC or disk diffusion test results it is important to ensure that the isolate is not *E. gallinarum* or *E. casseliflavus,* which may be erroneously perceived as *E. faecium* due to a positive arabinose test. The MGP (methyl-alpha-D-glucopyranoside) test or a motility test can be used to distinguish *E. gallinarum* /*E. casseliflavus* from *E. faecium* (MGP negative, non-motile). MALDI-TOF mass spectrometry is also useful for species identification of enterococci (13).

7.4.1 MIC determination

MIC determination may be performed by agar dilution, broth microdilution or gradient MIC methods.

Broth microdilution is performed according to the ISO standard 20776-1 as recommended by EUCAST. MIC determination with gradient tests is performed according to the manufacturer's instructions. Please note that MIC gradient strips are sometimes used with a higher inoculum (2.0 McFarland standard) on a rich medium (Brain Heart Infusion agar) to screen for vancomycin resistance but this analysis does not provide an MIC value.

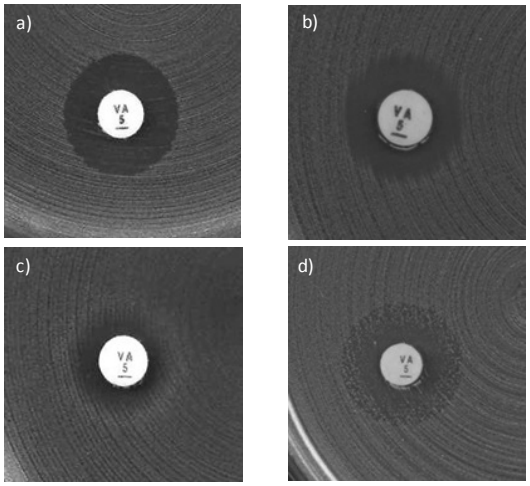
7.4.2 Disk diffusion testing

For disk diffusion the method specified by EUCAST must be followed meticulously. Inspect zones for fuzzy edges and/or microcolonies with transmitted light. Sharp zone edges indicate that the isolate is susceptible and isolates with sharp zones and zone diameters above the breakpoint can be reported as vancomycin susceptible.

Isolates with fuzzy zone edges or colonies within the zone (Figure 1) may be resistant regardless of zone size and should not be reported as susceptible without confirmation by MIC determination.

- Disk diffusion is performed according to the EUCAST disk diffusion methodology for non-fastidious organisms. Incubation for 24 h is needed in order to detect resistance in some isolates with inducible resistance.

Figure 1. Reading of vancomycin disk diffusion tests on *Enterococcus* spp.



a) Sharp zone edges and zone diameter ≥12 mm. Report as susceptible.

b-d) Fuzzy zone edges and/or colonies within the zone. Report as resistant regardless of zone diameter.

7.4.3 Breakpoint agars

Breakpoint agar tests with Brain Heart Infusion agar and 6 mg/l vancomycin are reliable for detection of *vanA*- and *vanB*-positive isolates. Breakpoint plates can be obtained from commercial manufacturers or made in-house. The breakpoint agar test is performed by application of 1 x 10^5 - 1 x 10^6 cfu (10 µl of a 0.5 McFarland suspension) on Brain Heart infusion agar with 6 mg/l vancomycin. Incubation for 24 h at 35±1°C in ambient air is needed in order to detect resistance in some isolates with inducible resistance. Growth of more than one colony is scored as a positive test.

7.4.4 Genotypic testing

Detection of vancomycin resistance by the use of PCR targeting *vanA* and *vanB* can also be performed using in-house or commercial methodologies (14-16).

7.4.5 Quality control

Appropriate strains for quality control of glycopeptide susceptibility tests are shown in table 2.

Table 2. Appropriate control strains for quality control of glycopeptide susceptibility tests.

Strain	Mechanism
E. faecalis ATCC 29212	Vancomycin-susceptible
E. faecalis ATCC 51299	Vancomycin-resistant (*vanB*)
E. faecium NCTC 12202	Vancomycin-resistant (*vanA*)

7.5 References

1. Depardieu F, Perichon B, Courvalin P. Detection of the *van* alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. J Clin Microbiol. 2004;42:5857-60.
2. Boyd DA, Willey BM, Fawcett D, Gillani N, Mulvey MR. Molecular characterization of *Enterococcus faecalis* N06-0364 with low-level vancomycin resistance harboring a novel D-Ala-D-Ser gene cluster, *vanL*. Antimicrob Agents Chemother. 2008;52:2667-72.
3. Xu X, Lin D, Yan G, Ye X, Wu S, Guo Y, Zhu D, Hu F, Zhang Y, Wang F, Jacoby GA, Wang M. *vanM*, a new glycopeptide resistance gene cluster found in *Enterococcus faecium*. Antimicrob Agents Chemother. 2010;54:4643-7.
4. Lebreton F, Depardieu F, Bourdon N, Fines-Guyon M, Berger P, Camiade S, Leclercq R, Courvalin P, Cattoir V. D-Ala-d-Ser VanN-type transferable vancomycin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother. 2011;55(10):4606-12.
5. Ramotar K, Woods W, Larocque L, Toye B. Comparison of phenotypic methods to identify enterococci intrinsically resistant to vancomycin (VanC VRE). Diagn Microbiol Infect Dis. 2000;36:119-24.
6. Mazuski JE. Vancomycin-resistant enterococcus: risk factors, surveillance, infections, and treatment. Surg Infect. 2008;9:567-71.
7. Tenover FC, McDonald LC. Vancomycin-resistant staphylococci and enterococci: epidemiology and control. Curr Opin Infect Dis. 2005;18:300-5.
8. Hayden MK, Trenholme GM, Schultz JE, Sahm DF. In vivo development of teicoplanin resistance in a VanB *Enterococcus faecium* isolate. J Infect Dis. 1993;167:1224-7.
9. Kawalec M, Gniadkowski M, Kedzierska J, Skotnicki A, Fiett J, Hryniewicz W. Selection of a teicoplanin-resistant *Enterococcus faecium* mutant during an outbreak caused by vancomycin-resistant enterococci with the *vanB* phenotype. J Clin Microbiol. 2001;39:4274-82.
10. Swenson JM, Clark NC, Sahm DF, Ferraro MJ, Doern G, Hindler J, Jorgensen JH, Pfaller MA, Reller LB, Weinstein MP, et al. Molecular characterization and multilaboratory evaluation of *Enterococcus faecalis* ATCC 51299 for quality control of screening tests for vancomycin and high-level aminoglycoside resistance in enterococci. J Clin Microbiol. 1995;33:3019-21.
11. Klare I, Fleige C, Geringer U, Witte W, Werner G. Performance of three chromogenic VRE screening agars, two Etest vancomycin protocols, and different microdilution methods in detecting *vanB* genotype *Enterococcus faecium* with varying vancomycin MICs. Diagn Microbiol Infect Dis. 2012;74:171-6.
12. Endtz HP, Van Den Braak N, Van Belkum A, Goessens WH, Kreft D, Stroebel AB, Verbrugh HA. Comparison of eight methods to detect vancomycin resistance in enterococci. J Clin Microbiol. 1998;36:592-4.
13. Fang H, Ohlsson AK, Ullberg M, Özenci V. Evaluation of species-specific PCR, Bruker MS, VITEK MS and the VITEK2 system for the identification of clinical *Enterococcus* isolates. Eur J Clin Microbiol Infect Dis. 2012; 31: 3073-7

14. Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995;33:1434.
15. Dahl KH, Simonsen GS, Olsvik O, Sundsfjord A. Heterogeneity in the *vanB* gene cluster of genomically diverse clinical strains of vancomycin-resistant enterococci. Antimicrob Agents Chemother. 1999;43:1105-10.
16. Gazin M, Lammens C, Goossens H, Malhotra-Kumar S; MOSAR WP2 Study Team. Evaluation of GeneOhm VanR and Xpert *vanA*/*vanB* molecular assays for the rapid detection of vancomycin-resistant enterococci. Eur J Clin Microbiol Infect Dis. 2012;31:273-6.

8. Penicillin non-susceptible *Streptococcus pneumoniae*

Importance of detection of resistance	
Required for antimicrobial susceptibility categorization	Yes
Infection control	No
Public health	Yes

8.1 Definition

S. pneumoniae isolates with reduced susceptibility to penicillin (MICs above those of the wild-type, i.e. >0.06 mg/L) due to the presence of modified penicillin-binding proteins (PBPs) with lower affinity for β-lactams.

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8.2 Clinical and/or epidemiological importance

S. pneumoniae is the most common cause of pneumonia worldwide. Morbidity and mortality are high and approximately three million people are estimated to die each year of pneumococcal infections. Low-grade penicillin non-susceptibility is associated with increased mortality when meningitis is treated with benzylpenicillin. In other infection types no increased mortality is observed with low-level resistance if higher dosages are given. Many countries carry out vaccination programmes against several pneumococcal serotypes, and this may also affect resistance levels observed in invasive isolates (1). However, penicillin non-susceptible *S. pneumoniae* remain a major clinical problem from a public health point of view, although these microorganisms are not associated with spread in healthcare institutions, unlike many of the other pathogens described in this document.

8.3 Mechanism of resistance

S. pneumoniae contains six PBPs, of which PBP 2x is the primary target of penicillin (2). The presence of "mosaic genes" encoding low-affinity PBPs is the result of horizontal gene transfer from commensal viridans streptococci (2). The level of β-lactam resistance depends not only on low-affinity mosaic PBPs present in the isolate, but also on modification of the specific PBPs that are essential for *S. pneumoniae* (3). Strains with MICs of benzylpenicillin in the range 0.12 to 2 mg/l are considered susceptible in non-meningitis infections when a higher dose of penicillin is used, whereas for meningitis such strains must always be reported as resistant (4).

8.4 Recommended methods for detection of penicillin non-susceptible *S. pneumoniae*

Penicillin non-susceptibility can be detected phenotypically by MIC or disk diffusion methods.

8.4.1 Disk diffusion method

The disk diffusion method with 1µg oxacillin disks is an effective screening method for the detection of penicillin non-susceptible pneumococci (5, 6, 7). The method is very sensitive, but is not highly specific as strains with zone diameters of ≤19 mm may have variable susceptibility to benzylpenicillin, and the benzylpenicillin MIC should be determined for all isolates that are non-susceptible with the screening method (7).

For β-lactams other than benzylpenicillin the oxacillin zone diameter can be used to predict susceptibility as in Table 1.

Table 1. Screening for β-lactam resistance in *S. pneumoniae*

Zone diameter (mm) with oxacillin (1µg)	**Antimicrobial agents**	**Further testing and/or interpretation**
≥ 20 mm	All β -lactam agents for which clinical breakpoints are listed (including those with "Note")	Report susceptible irrespective of clinical indication, except for cefaclor, which if reported, should be reported intermediate
< 20 mm*	Benzylpenicillin (meningitis) and phenoxymethylpenicillin (all indications)	Report resistant.
	Ampicillin, amoxicillin and piperacillin (with and without β -lactamase inhibitor), cefotaxime, ceftriaxone, ceftaroline and cefepime.	Oxacillin zone diameter ≥ 8 mm: Report susceptible. In meningitis: confirm by determining the MIC of the agent considered for clinical use
		Oxacillin zone diameter < 8 mm: determine the MIC of the β -lactam agent intended for clinical use but for ampicillin, amoxicillin and piperacillin (without and with β -lactamase inhibitor) infer susceptibility from the MIC of ampicillin.
	Other β -lactam agents (including benzylpenicillin for infections other than meningitis)	Test by an MIC method for the agent considered for clinical use and interpret according to the clinical breakpoints

*Oxacillin 1 µg <20 mm: Always determine the MIC of benzylpenicillin but do not delay reporting of other β-lactams as recommended above.

8.4.2 Clinical breakpoints

The penicillin breakpoints were primarily designed to ensure the success of therapy for pneumococcal meningitis. However, clinical studies demonstrated that the outcome of pneumococcal pneumonia caused by strains with intermediate susceptibility to penicillin and treated with parenteral penicillin was no different to that for patients treated with other agents. Considering microbiological, pharmacokinetic and pharmacodynamic data, the clinical breakpoints for

benzylpenicillin for non-meningitis isolates were revisited (3) and current EUCAST breakpoints are as listed in Table 2.

Table 2. Reporting of benzylpenicillin susceptibility in meningitis and non-meningitis.

Indications	**MIC breakpoint (mg/L)**		**Notes**
	S ≤	R >	
Benzylpenicillin (non-meningitis)	0.06	2	**In pneumonia**, when a dose of 1.2 g x 4 is used, isolates with **MIC ≤0.5 mg/L** should be regarded as susceptible to benzylpenicillin. **In pneumonia**, when a dose of 2.4 g x 4 or 1.2 g x 6 is used, isolates with **MIC ≤1 mg/L** should be regarded as susceptible to benzylpenicillin. **In pneumonia**, when a dose of 2.4 g x 6 is used, isolates with **MIC ≤2 mg/L** should be regarded as susceptible.
Benzylpenicillin (meningitis)	0.06	0.06	

Note: 1.2 g of benzylpenicillin is equal to 2 MU (million units) of benzylpenicillin

8.4.3 Quality control

Appropriate strains for quality control of benzylpenicillin susceptibility tests are shown in table 3.

Table 3. Appropriate control strains for quality control of benzylpenicillin susceptibility tests.

Strain	**Mechanism**
S. pneumoniae ATCC 49619	Mosaic PBP, benzylpenicillin MIC 0.5 mg/L

8.5 References

1. Dagan R. Impact of pneumococcal conjugate vaccine on infections caused by antibiotic-resistant Streptococcus pneumoniae.Clin Microbiol Infect. 2009;15 (Suppl 3):16-20.
2. Hakenbeck R, Kaminski K, König A, van der Linden M, Paik J, Reichmann P, Zähner D. Penicillin-binding proteins in beta-lactam-resistant *Streptococcus pneumoniae*. Microb Drug Resist 1999; 5: 91-99.
3. Grebe T, Hakenbeck R. Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of β-lactam antibiotics. Antimicrob Agents Chemother 1996; 40: 829-834.
4. Weinstein MP, Klugman KP, Jones RN. Rationale for revised penicillin susceptibility breakpoints versus *Streptococcus pneumoniae*: Coping with antimicrobial susceptibility in an era of resistance. Clin Infect Dis 2009; 48: 1596 – 1600.
5. Dixon JMS, Lipinski AE, Graham MEP. Detection and prevalence of pneumococci with increased resistance to penicillin. Can Med Assoc J 1977; 117: 1159-61.

6. Swenson JM, Hill BC, Thornsberry C. Screening pneumococci for penicillin resistance. J Clin Microbiol 1986; 24: 749-52.
7. Jetté LP and C Sinave. Use of an oxacillin disk screening test for detection of penicillin- and ceftriaxone-resistant pneumococci. J Clin Microbiol 1999; 37: 1178-81.

9. Transparency declarations

CGG: conference support and research collaboration with AB Biodisk (later purchased by bioMérieux), has received speaker's honorarium from BioRad and Liofilchem, member of the EUCAST Steering Committee and the EARS-Net Coordination Group
LMM: has been consultant for Wyeth and Pfizer, has presented lectures for Wyeth, Merck, Pfizer, Janssen-Cilag and Astra-Zeneca, and has received research grants from Merck, Wyeth, Janssen-Cilag and Astra-Zeneca, member of the EUCAST Steering Committee
RC: has participated in educational programs sponsored by Siemens, BioRad and Liofilchem and in research projects founded by BD, BioRad and Liofilchem, chairman of EUCAST
RS: scientific advisor for Novartis (terminated 2012), consultant for bioMérieux and Pfizer, has received speaker's honorarium from Cepheid and Becton Dickinson member of the EUCAST Steering Committee
SS: no conflict of interests to declare
YG: no conflict of interests to declare
PN: has taken patent for the Carba NP and ESBL NDP tests on behalf of the INSERM (Paris, France)
MW: no conflict of interests to declare
VM: no conflict of interests to declare
GSS: member of the EARS-Net Coordination Group
HS: member of the EARS-Net Coordination Group
JCS: no conflicts of interest to declare.
MG: has participated in an educational program sponsored by Liofilchem